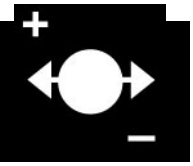
RIBONUCLEIC ACIDS: CAPILLARY ELECTROPHORESIS



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Introduction

With the introduction of capillary electrophoresis (CE), a new generation of electrophoretic techniques has seen the light of day. The scientific literature today describes a large number of applications of this powerful analytical technique in the analysis of nucleic acids. For nucleic acids, as for most other analytes, CE offers significant advantages over many of the conventional electrophoretic techniques. In general, CE is characterized by short analysis time, high resolution, accuracy and reproducibility, quantitative online detection and automation. The small sample volumes required and the extreme sensitivity CE offers, represent a large analytical potential for samples of biological origin. The fundamental analytical and operational parameters for the separation of nucleic acids by CE were identified around 1990. A decade later, CE is considered a fully developed technology for the analysis of DNA. The rapid development of this application of CE seems to have been driven by the many practical applications of electrophoretic separation and detection of DNA in both basic and applied science.

The first reported analysis of RNA by CE was published in 1993 by Reyes-Engel *et al*. and describes the separation and quantification of a specific messenger RNA by capillary zone electrophoresis. To date, only a limited number of articles have been published which focus on the application of CE in the analysis of RNA. The reason for this is not obvious, considering the widespread use of conventional gel electrophoresis of RNA throughout the biomedical scientific field. The fact that RNA, in many respects, displays similar characteristics as DNA, should constitute the basis for significant efforts in the development of RNA analyses based on CE. However, the scientific literature holds promise for a substantial increase in the use of CE in RNA analyses. The following sections intend to give a basic introduction to CE of RNA, with emphasis on important analytical and operational parameters in the analyses. Finally, examples from a diverse group of applications are presented.

Capillary Electrophoresis of RNA

In general, electrophoretic separation of RNA is based on the differences in electrophoretic mobilities of the analytes. As in conventional electrophoresis, the rate of migration of a RNA molecule in CE depends on the mass and the dimensions of the molecule, the charge carried, the applied current and the resistance of the medium. In an electric field, at moderate pH, negatively charged RNA migrates toward the anode. A number of parameters affect the separation of RNA in CE (see below). CE of RNA can be divided into two separate categories based on the principle by which the molecules are separated: capillary zone electrophoresis (CZE) and capillary gel electrophoresis (CGE). In CZE, the RNA molecules are mainly separated by their charge to mass ratio. From the fact that nucleic acids larger than a few nucleotide units have approximately identical charge to mass ratio, CZE provides little or no separation power. Consequently, only single RNA species can be identified by this technique, unless multiple labelling is being used. In CGE, the RNA molecules are separated mainly by their molecular dimensions, i.e., the ability of the different analytes to migrate through a gel matrix. CGE is by far the most common technique for RNA analyses. A description of CGE of RNA is given in the following section.

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Capillary Gel Electrophoresis of RNA

Analytical parameters of significance for the separation of DNA by CGE, including gel polymer concentration, electrical field strength and temperature, have been investigated and optimized for the analysis of RNA molecules ranging from oligomers (10 to 40 bases) to several kilobases (**Figure 1**).

RNA Migration in Capillary Gel Electrophoresis

In conventional gel electrophoresis, the migration of a RNA molecule is inversely related to the $\log_{10}$ molecular mass. However, base composition (primary structure) and secondary structure can affect this relationship. In CGE, separation is achieved because large molecules move more slowly through the gel than small molecules. Separation within a given RNA molecular range is obtained by selecting a gel of

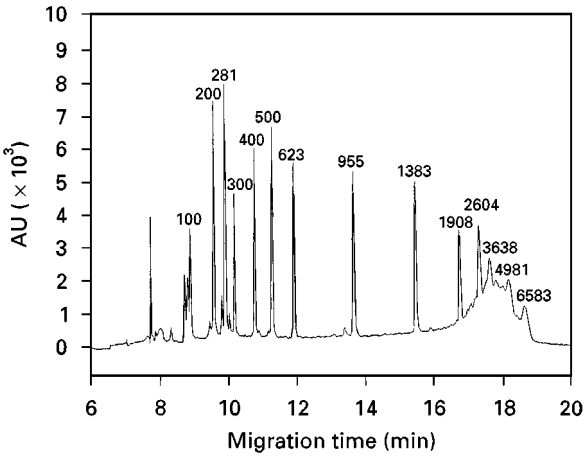

Figure 1 Electropherogram of RNA molecular-mass marker. The sample was denatured, injected at 300 V cm^{-1} for 10 s and subjected to CGE at 200 V cm^{-1} in 1 × TBE/8 mol L^{-1} urea containing 0.3% HPMC. AU, arbitrary units. (Reprinted from Skeidsvoll J and Ueland PM (1996) Analysis of RNA by capillary electrophoresis. *Electrophoresis* 17: 1512–1517. Copyright 1996, with permission from Wiley-VCH Verlag GmbH.)

appropriate pore size. Experiments have demonstrated that CGE of higher molecular mass RNA (in the range from 100 bases to more than 6 kb) to a large extent resembles CGE of single-stranded DNA. An interesting finding is that RNA and single-stranded DNA of identical length display different migration when co-analysed under completely denaturing conditions, DNA having a slightly higher migration rate than RNA (**Figure 2**). The shift in migration for DNA vs. RNA is found constant for molecules ranging from 100 to approximately 1000 bases. The phenomenon is explained by the higher charge to mass ratio of single-stranded DNA.

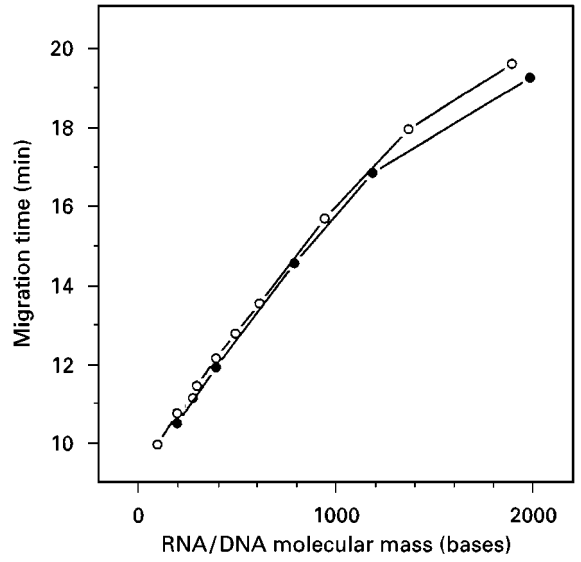

Figure 2 Comparison of migration of RNA and single-stranded DNA. A molecular-mass marker containing RNA and DNA components was denaturated by pre-incubation at 95°C for 3 min in the presence of 80% formamide and subjected to electrophoresis in a separation buffer containing 8 mol L^{-1} urea and 0.3% HPMC. Migration time is plotted versus molecular mass for RNA (○) and DNA (●). (Reprinted from Skeidsvoll J and Ueland PM (1996) Analysis of RNA by capillary electrophoresis. *Electrophoresis* 17: 1512–1517. Copyright 1996, with permission form Wiley-VCH Verlag GmbH.)

An inherent property of the (single-stranded) RNA molecule is the potential to form secondary structures or intramolecular and intermolecular hydrogen bonds. To what extent the reaction takes place is primarily a function of the RNA sequence. The predictable determination of RNA molecular mass is essential in most RNA techniques based on electrophoretic separation. Consequently, in order to prevent unpredictable migration of RNA due to the formation of secondary structures, CE should be carried out under completely denaturing conditions. Such conditions can be accomplished through optimization of physical and/or chemical parameters. For example, heating the sample in the presence of a denaturant prior to electrophoresis and addition of a denaturant in the electrophoresis and separation buffers combined with high temperature during electrophoresis should have a strong denaturing effect. Denaturants are chemical compounds that disrupt hydrogen bonds. The most commonly used denaturant, urea, is often added to the separation buffer in very high concentrations (up to 8 mol L^{-1}). Despite an extensive use of buffer additives, data from both conventional RNA gel electrophoresis and CE of RNA indicate that even the presence of 8 mol L^{-1} urea in the separation buffer is not sufficient to completely disrupt intramolecular or intermolecular hydrogen bonds. Addition of the stronger denaturant formamide in concentrations up to 30% (in addition to 3.5 mol L^{-1} urea) and performing CE at 60°C has been necessary to disrupt extensive secondary structures in a hammerhead ribozyme (37 nucleotides) and to provide appropriate separation from its substrate (17 nucleotides). In addition, a decrease in ionic (cationic) strength and an increase in pH are known to have a denaturing effect on RNA. Common problems related to inefficient separation, detection and identification of RNA in CE, probably result from incomplete denaturation of RNA.

Important Analytical and Operational Parameters

From the comprehensive scientific literature describing CE of nucleic acids, it is obvious that operational parameters like capillary dimensions (m) electrical field

strength (E, V cm^{-1}) and temperature (t, °C) have to be chosen carefully to optimize the separation of RNA.

In most applications of CE in RNA analyses, the electroosmotic flow is eliminated through the use of surface-modified (coated) capillaries. This considerably simplifies the experimental design and leaves the scientist with a limited number of variable analytical and operational parameters.

Buffer Composition

In general, all buffer systems that are used for CZE can also be used for CGE. The most common buffers are the Tris-borate buffers (i.e., TBE) with a pH range of 7.5–9.0. Buffer additives like methanol and acetonitrile are used in separation buffers optimized for low-molecular-mass RNA. Urea and formamide are mainly added as denaturants. Moreover, the addition of urea to the separation buffer has been found to increase the resolution of RNA (except for oligoribonucleotides less than 5 bases).

Gel-forming Polymers

A number of different gel-forming polymers have successfully been used in both DNA and RNA separations by CE. The separation matrices comprise both cross-linked gel polymers like polyacrylamide and noncross-linked gel polymers like linear polyacrylamide and cellulose derivatives. Through the optimization of composition and concentration, noncross-linked polymers have now taken over as the predominant separation matrices for most RNA analyses. These materials have demonstrated significant advantages over cross-linked polymers, including ease of preparation and use, physical stability and uncomplicated washing and refilling procedures between analyses. The resolving power of these gels mainly depends on the concentration of the dissolved polymer – dilute gels are used for high-molecular-mass RNA molecules and more concentrated gels for low-molecular-mass RNA.

A systematic study of the electrophoretic separation of RNA at different concentrations of a noncross-linked polymer gel demonstrated that high concentrations ($>0.3\%$) hydroxypropylmethylcellulose (HPMC) were optimal for the separation of RNA less than 1000 bases and low concentrations were optimal for the separation of higher molecular-mass RNA. The results are consistent with data from the separation of DNA by CE.

A number of separation matrices, optimized for different ranges of RNA molecular mass, are commercially available. Additionally, matrices are available which contain denaturants.

Electrical Field Strength

Electrical field strength is recognized as an important operational parameter in CE of RNA. An increase in electrical field strength is found to result in a logarithmic decline in migration times. Efficiency, N (number of theoretical plates) and resolution, R_s, are found to have a more complex relation to the electrical field strength, although a clear tendency towards a decline in both parameters with increased electrical field strength has been demonstrated. In general, low electrical field strengths are preferable for the optimal separation of RNA molecules larger than 100 bases. With the increase in electrical field strength, an increased current will result in the production of heat (Joule heating), which, if excessive, adversely affects the separation by causing broadening of the migrating zones.

Temperature

Temperature, an important analytical and operational parameter, influences both total analysis time and system efficiency. The effect is mainly mediated by a decrease in the separation buffer viscosity. A linear decrease in migration time for RNA molecules ranging from 200 to 2000 bases has been observed for temperatures ranging from 20 to 50°C. The separation efficiency and resolution were found essentially constant over the temperature range. In addition, temperature is a parameter of significant importance in the CE of RNA due to its denaturing effect on intra- and intermolecular hydrogen bonds.

Quantitative Aspects

For a general description of the quantitative aspects of injection in CE, see 'DNA: Capillary Electrophoresis'. Electrokinetic injection is the most common injection mode for RNA in CE. In order to obtain quantitative data, an external reference should be added to or co-injected with the RNA sample. Ideally, the external reference should resemble the sample of interest, but be readily identifiable. Hydrodynamic injection is often used in experiments for the determination of reaction kinetics or in studies of enzymatic activity. Hydrodynamic injection provides representative samples for analysis.

UV absorbance is the most common detection principle for RNA in CE. Despite its general usefulness, the technique suffers from low sensitivity as compared to other detection principles (e.g., laser-nduced fluorescence) and represents a limiting factor in some RNA analyses. Detection of RNA based on (laser-induced) fluorescence confers the selectivity

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and sensitivity required for a number of analyses where the concentration of analytes is low. However, this detection principle normally requires the covalent attachment of fluorophores to target molecule(s) or fluorogenic buffer additives.

Applications

The application of CE to RNA includes a diverse group of analyses, which often includes one or a combination of the following elements:

- Characterization of RNA molecular dimensions (mass or spatial structure).
- Characterization of RNA sequence.
- Characterization of RNA reaction kinetics.
- Characterization of RNA-binding constants.

An example of a group of CE-based RNA analyses that combines more than one of these elements is the hybridization techniques, which both rely on molecular mass determination and sequence-specific detection of the RNA of interest. In the applications described, RNA samples originate either from chemical synthesis (oligoribonucleotides) or are extracted from biological material. The last group comprise RNA of eukaryotic, prokaryotic and viral origin.

Characterization of RNA Molecular Dimensions (Mass or Spatial Structure)

Capillary electrophoresis analysis of synthetic short-chain oligoribonucletides (Figure 3) Thirty synthetic oligoribonucleotides, ranging from 3 to 18 nucleotides were analysed by CE in a nondenaturing noncross-linked gel polymer. An equation was developed, based on the experimental data which, under fixed conditions, accounts for the influence of charge to mass ratio (i.e., net charge and base composition) on migration time. High resolution (1 nucleotide unit) was obtained for homologous series of oligoribonucleotides, and, to some extent, for groups of oligoribonucleotides of identical length, but different sequence.

CGE is often used to determine the quality of chemically synthesized oligoribonucleotides and can be used in conjunction with HPLC to develop an effective method for the purification of crude oligonucleotide solutions.

Low-molecular-mass RNA fingerprinting of bacteria by capillary electrophoresis RNA profiling provides a direct genotypic fingerprint technique for the identification and classification of bacteria by generating an electropherogram including three groups of molecules of taxonomic significance, small tRNAs, large tRNAs, and 5S rRNA (ranging from 70 to 135 nucleotides). The technique is of particular importance for molecular ecology and taxonomic studies, and can also be applied directly to analyses of environmental samples. CGE using both noncross-linked polymer gels (HPMC) and cross-linked polyacrylamide gels have been investigated and optimized for their applicability in the separation of this class of RNA molecules. Good resolution was obtained only for small tRNAs up to approximately 80 nucleotides using cross-linked gels, larger tRNAs and 5S rRNA could not be resolved with this experimental set-up. The use of noncross-linked polymer gels resolved tRNAs and 5S rRNA under nondenaturing conditions, even when they possessed only different secondary structure or small differences in length (1–5 nucleotides). CE using HPMC in the separation buffer resulted in both good peak resolution and reproducibility and was suitable for routine fingerprinting of bacterial low-molecular-mass RNA.

Investigation of intracellular metabolism of a stabilized ribozyme by CGE after uptake by transfection or as free ribozyme CGE has been used to investigate

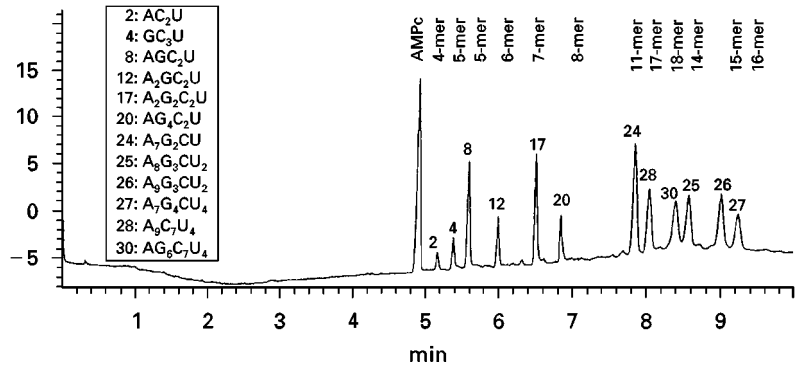

Figure 3 CGE analysis of a mixture of 12 oligoribonucleotides from 4 to 18 units under nondenaturing conditions. (Reprinted from Kolesar JM, Allen PG and Doran CM (1997) Direct quantification of HIV-1 RNA by capillary electrophoresis with laser-induced fluorescence. *Electrophoresis* 697: 189–194. Copyright 1997, with permission from Elsevier Science.)

cellular uptake and degradation of a fluorescein labelled chemically stabilized ribozyme (37-mer). After internalization by transfection or uptake of free ribozyme, electrophoretic peaks of intact ribozyme and different degradation products were easily resolved and the amount of intracellular intact ribozyme quantified. Using laser-induced fluorescence for detection, the method offered extreme sensitivity with estimated limit of detection: 10 and 200 pmol L^{-1} ribozyme from cell extracts and cell medium, respectively.

A third example include the direct quantification (by UV absorbance measurements) of HIV-1 RNA in human plasma by CZE.

Characterization of RNA Sequence

An important and diverse group of analytical RNA techniques is based on sequence-specific hybridization between two single-stranded nucleic acids and the electrophoretic separation, detection and quantification of the intermolecular reaction product (hybrid). Consequently, the analyses involves characterization of RNA in two dimensions, size and sequence. The Northern (RNA) blotting technique, the nuclease- (S1 or RNase) protection assays and other RNA-hybridization techniques play an important role in the qualitative and quantitative analysis of all classes of RNA in biological systems. The techniques often involve use of radioisotope labels in detection.

CE-based hybridization analyses of RNA has been successfully demonstrated for a number of applications. In general, the hybridization reactions are carried out pre-column (in solution) and the separation and detection of the hybrids on-column. It is demanding to transfer the conventional hybridization techniques to the capillary format and important challenges are related to the development of selective and compatible conditions for both the pre-column and on-column elements of the analyses. Additionally, the low sample volumes injected in CE represent significant analytical and instrumental challenges.

Direct quantification of a specific messenger RNA by capillary zone electrophoresis Total RNA was isolated from whole blood and hybridized with a biotinylated oligonucleotide specific for a receptor mRNA (angiotensin II). The hybrid was first captured on streptavidin-conjugated magnetic beads, then eluted and finally quantified by UV absorbance in CZE. Using this procedure, quantification of the expression of low expressed genes is easy and fast and subject to two limiting factors: the specificity of the capturing oligonucleotide or probe selected and the amount of total RNA. The procedure represents an nonradioactive alternative to conventional RNA analyses like Northern blotting, RT-PCR or the nuclease- (S1 or RNase) protection assays.

Direct quantification of HIV-1 RNA by capillary electrophoresis with laser-induced fluorescence (LIF) detection (Figure 4) A hybridization method using a HIV-specific probe with analysis by CE–LIF was developed. Plasma samples from HIV-seropositive patients were lysed to obtain RNA, hybridized with a fluorescein-labelled HIV-specific DNA probe, digested by a specific RNase to remove nonhybridized RNA and analysed by CE-LIF in presence of the fluorescent intercalator thiazole orange (TO). 19 fg (corresponding to 1710 copies per mL of starting plasma) of HIV RNA was quantitatively detected. The technique, analogous to the conventional RNase protection assay, takes advantage of signal amplification by using the RNA-binding fluorescent intercalator TO. Calibration is done through the analysis of a fluorescein-labelled RNA marker. The actual approach appears to be an efficient, sensitive and reliable method to specifically and quantitatively analyse RNA from a variety of sources.

Detection of oligonucleotide N3′–P5′ phosphoramidate/RNA duplexes with capillary gel electrophoresis The DNA analogues N3′–P5′ phosphoramidates (3′-phosphoramidates) has demonstrated favourable properties as hybridization probes, including high melting temperature of duplexes with RNA and high reaction rate at low ionic strengths. The RNA hybridization technique takes advantage of the 3′-phosphoramidate oligomer properties as hybridization probes through duplex formation with short complementary strands of RNA of identical length (9 nucleotides). Hybrids were found to have unique relative mobilities in CGE, compared to the reactants. The ability of CGE to detect the presence of, and to discriminate between, perfect duplexes and duplexes that contained a base mismatch were demonstrated under routine electrophoretic running conditions. In conclusion, the study indicates that 3′-phosphoramidate oligonucleotides may have application in nucleic acid based diagnostics.

Characterization of RNA Reaction Kinetics

Current commercial CE instrumentation offers the scientist operational functions like thermostated sample compartments, automatic sampling and thermostated analyses. These functions increase the potential of CE technique developments, compared with most conventional gel electrophoresis systems, and are especially useful in studies of reaction kinetics, for the determination of association and dissociation constants and in enzymatic assays.

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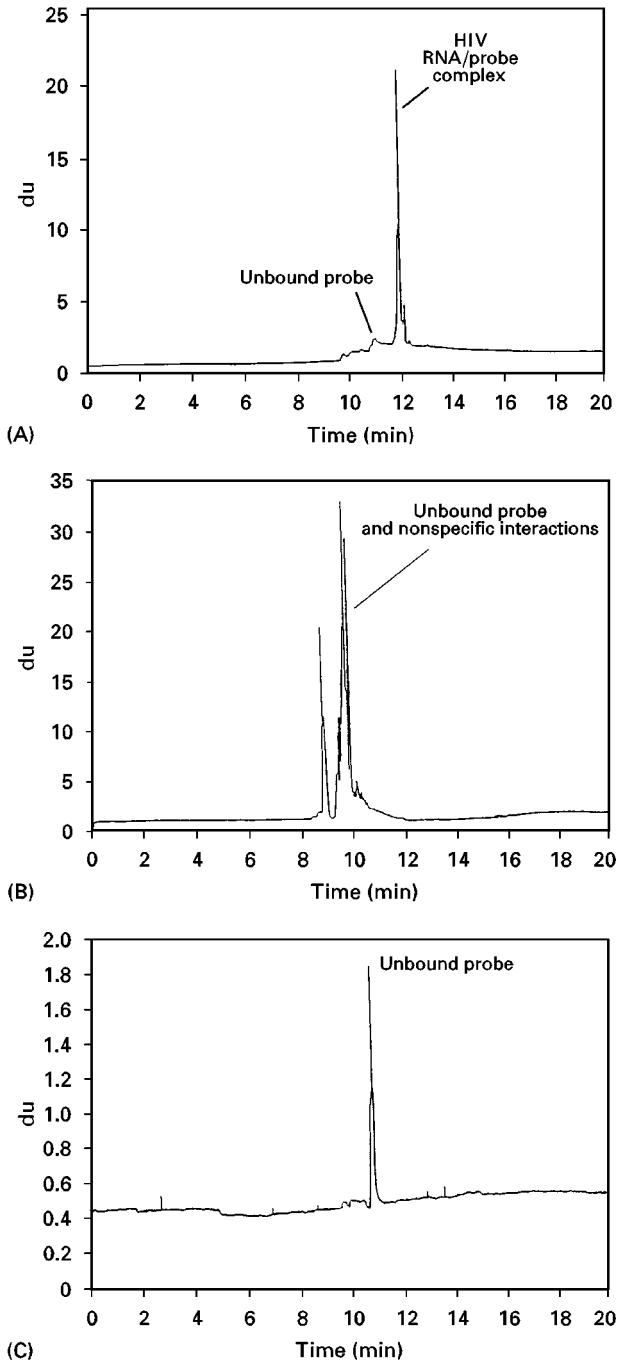

Figure 4 Electropherogram from a hybridization experiment. RNA samples obtained from a HIV-seropositive patient and a seronegative volunteer were hybridized with a HIV-specific probe and analysed by CGE: (A) HIV RNA/probe complex (HIV-positive patient); (B) seronegative volunteer; (C) negative control containing all reaction components except RNA. (Reprinted from Kolesar JM, Allen PG and Doran CM (1997) Direct quantification of HIV-1 RNA by capillary electrophoresis with laser-induced fluorescence, *Journal of Chromatography B* 697: 189–194. Copyright 1997, with permission form Elsevier Science.)

A thermostated and closed sample vial and a computer-controlled injection system is equivalent to a chemical reaction chamber and an automatic sampling operation, respectively. CE has developed into an effective technique, for example, determination of apparent equilibrium constants for molecular association in solution. Examples of CE being used in the characterization of RNA reaction kinetics are described below.

Determination of the catalytic activity of a hammerhead ribozyme (Figure 5) Ribozymes are sequences of catalytic RNA. The catalytic activity of a synthetic hammerhead ribozyme (37-mer), i.e., the hydrolysis of its RNA substrate (17-mer), has been determined by regular injection from the reaction vial. Kinetic parameters like k_m and k_{cat} were calculated from the integrated area of the decreasing substrate peak. Experimental conditions compatible with both ribozyme activity and CE separation of ribozyme and substrate were determined by careful optimization of the reaction mixture (sample) and the separation buffer. A combination of thermal and chemical denaturation was necessary to separate the oligoribonucleotides. Kinetic analyses were performed in the range where

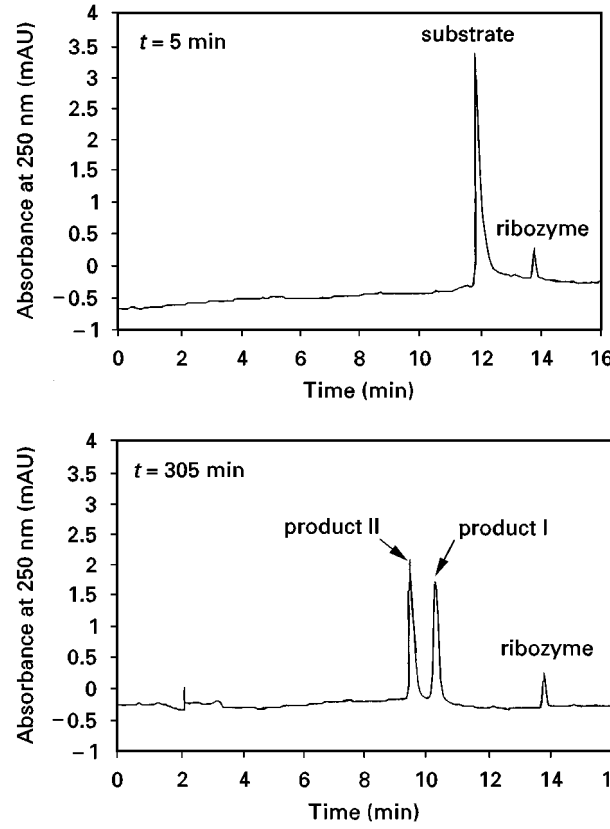

Figure 5 Typical electropherograms demonstrating different stages in a ribozyme-mediated catalytic breakdown of a RNA oligonucleotides substrate. (Reprinted from Saevels J, Schepdael AV and Hoogmartens J (1999) Capillary electrophoresis of RNA oligonucleotides: catalytic activity of a hammerhead ribozyme. *Analytical Biochemistry* 266: 93–101. Copyright 1999, with permission from Academic Press.)

the substrate exhibited linear detector response. RNA detection by UV absorbance was found to be a limiting factor in the Michaelis–Menten analysis.

Characterization of pre-tRNA maturation by RNase using capillary gel electrophoresis A CGE-based technique has been developed in order to characterize the reaction kinetics and mechanism for maturation of a set of pre-tRNA^{fMet} mutants. At all steps of the study of RNase P, including the preparation of the pre-tRNA (quality), the kinetic analysis and the control and yield of the purification steps, CGE was found appropriate and reliable.

Analysis of a ribonuclease H digestion of N3′–P5′ phosphoramidate–RNA duplexes by capillary gel electrophoresis The activity of a ribonuclease H (RNase H)-mediated RNA hydrolysis of duplexes formed by oligodeoxyribonucleotides or their analogue, N3′–P5′ phosphoramidates and complementary RNA strands, have been investigated. The enzymatic assay conditions were carefully optimized enabling sampling directly from the reaction mixture. CGE electropherograms revealed that RNA–N3′–P5′ phosphoramidates duplexes remained intact and therefore did not appear to be a substrate for RNase H.

Conclusion

Today, CE of nucleic acids has become an important analytical technique for biochemists and molecular biologists and the scientific studies described here clearly illustrate the applicability of CE in the analysis of RNA. Through efficient separations of RNA molecules ranging from a few bases to several kilobases, the specific and sensitive detection of RNA sequences and the study of RNA reaction kinetics, scientists have taken advantage of the prominent characteristics of CE. Compared to the analysis of DNA, additional challenges exist in the analysis of RNA, challenges mainly related to RNA stability and conformation. However, efforts should be made to overcome problems related to inefficient separation, identification and detection of RNA in CE. Extended insight into these phenomena will realize the inherent potential of CE for a diversity of RNA analyses.

Further Reading

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See **III / DEOXYRIBONUCLEIC ACID PROFILING: Capillary Electrophoresis**

SELECTIVITY OF IMPRINTED POLYMERS: AFFINITY SEPARATION

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Ever since the discovery of antibodies and receptors, and their remarkable selectivities for almost any given chemical structure, scientists have been intrigued by the quest of mimicking their properties in synthetic or semisynthetic systems. A material carrying a selective preference for one ligand in comparison with other structurally similar compounds would be of outstanding use in a wide variety of situations, extending from molecular transportation, via analysis, to catalysis and synthesis. A multitude of sophisticated approaches have also been developed over the years, with the objective of controlling ligand binding to an artificial receptor.